0273-2289/03/111/0129/$25.00

Fermentation Process Optimization of Recombinant *Saccharomyces cerevisiae* for the Production of Human Interferon-α2a

JU CHU,* SILIANG ZHANG, AND YINGPING ZHUANG

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, P.R. China, E-mail: juchu@online.sh.cn

Received August 2002; Revised April 2003; Accepted June 2003

Abstract

The effects of different culture conditions on the expression level of human interferon-α2a (IFN-α2a) by using recombinant yeast were investigated in a 2.6-L jar fermentor. Appropriate supplement of glucose and the maintenance of residual glucose at a low level resulted in the reduction of ethanol formation and enhancement of the bioactivity of IFN-α2a to 4.9×10^6 from 3.1×10^6 IU/mL. When adenine was added evenly for 10–20 h of fermentation into the basal culture medium at a speed of 2 μg/mL of medium/h, OD_{600} was greatly increased to 24, and the protein increased to 276 mg/L. The content of ethanol generated was also reduced tremendously during the process, and as a result, 1.3×10^7 IU/mL of biologic activity was achieved. In the expression phase, pH had an important impact on expression level, which should be controlled at 5.5.

Index Entries: Interferon-α2a; *Saccharomyces cerevisiae*; cultivation; recombinant yeast; fed-batch fermentation; adenine.

Introduction

Human interferon-α2a (IFN-α2a) is an antivirus and anticancer medicine applied extensively around the world. It is a single-strand polypeptide composed of 165 amino acids, and it has a mol wt of 19,219 Daltons. IFN-α2a is capable of repressing the growth of various human tumors, inhibiting the transcription of the codon of viral nucleic acid, and disassembling the viral RNA. It is a multifunctional cell factor that regulates

*Author to whom all correspondence and reprint requests should be addressed.

immunity and possesses the curative effects of hepatitis C, leukemia, and Kaposi's sarcoma (related to acquired immunodeficiency syndrome) *(1)*.

Because of the low cost of production and high yields (10^5–10^6 U/mL of interferon fermentation), yeast or bacteria used as IFN-producing strain was considered to be far more advantageous than cell culture *(2)*. About 2×10^5 U/mL of human leukocyte IFN produced in *Escherichia coli* has been reported *(3)*. Yields of 3×10^6 U/mL of human IFN-α have also been achieved in yeast *(4)*.

Saccharomyces cerevisiae is an attractive host for the production of foreign protein and has been used extensively in baking and brewing industries. It has a regulation mechanism of gene expression that is more mature than that of *E. coli*, and it has the strong capability of excretion and postprocessing of genetic products. Consequently, being an expression system for genetic engineering, *S. cerevisiae* has been used successfully in the expression of foreign genes *(5)*. IFN-α2a is the only product expressed by the eukaryotic system at Shanghai Wanxing Bio-Pharmaceutical (P.R. China), and the issues of clinical side effects have been resolved. The work presented here involves the growth of this recombinant *S. cerevisiae* to high cell density and the concurrent production of high concentrations of IFN-α2a in fed-batch fermentation with a 2.6-L fermentor equipped with multiparameter monitoring and control devices.

Materials and Methods

Microorganism

The engineered yeast DCO4(Cir^0,pHC11-IFNαA1) used in the fermentation was supplied by Shanghai Wanxing Bio-Pharmaceutical. It was constructed by transforming the recombinant plasmid pHC11-IFNαA1 into the *Saccharomyces carlsbergensis* DCO4(Cir^0,MATa, ade1, leu2-04) *(6)*. The plasmid was constructed by inserting the human αA IFN gene and the secretion expression unit into the highly stabilized vector pHC11. The yeast phosphoglycerate kinase gene promotor was applied to the recombinant plasmid.

Media and Culture Conditions

SD seed medium consisted of 6.7 g/L of YNB (yeast nitrogen base without amino acids; Difco, Detroit, MI), 20 g/L of glucose, and 40 μg/mL of adenine, and the pH was unadjusted. Yeast fermentation medium consisted of 15 g/L of yeast extract (Difco), 20 g/L of polypeptone (made in Japan), 20 g/L of glucose, 2 g/L of Tween-20, and 20 μg/mL of adenine, and the pH was unadjusted. One milliliter of inoculum was transferred from a lyophilized glycerol tube to a 500-mL Erlenmeyer flask containing 75 mL of seed medium and cultivated on a rotary shaker at 30°C at 240 rpm for 18 h. The seed culture was then transferred to a 250-mL Erlenmeyer flask containing 25 mL of fermentation medium or a 2.6-L Marubishi MD-250

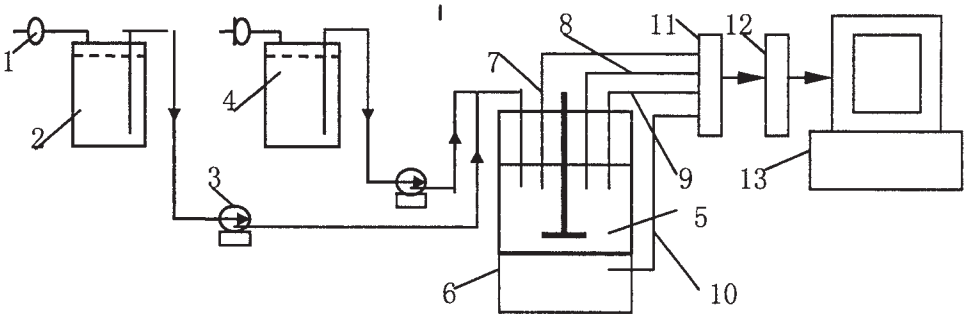

Fig. 1. Setup of fed-batch fermentation system for IFN-α2a production. 1, Air filter; 2, glucose vessel; 3, peristaltic pump; 4, feed vessel; 5, fermentor; 6, magnetic driver; 7, temperature sensor; 8, pH electrode; 9, DO probe; 10, agitation speed transducer; 11, signal amplifier; 12, A/D converter; 13, computer for data logging.

fermentor containing 1.5 L of fermentation medium with 5% inoculum and cultivated for 36 h.

Fermentation Equipment

A 2.6-L jar fermentor (model MD-250, manufactured by Marubishi, Japan) was applied. It was equipped with sensors for dissolved oxygen tension (DO), pH, temperature, and agitation speed monitoring and control. The signals were amplified and converted by an analog/digital (A/D) converter before they were input into a PC, and the fermentation data were logged and displayed by the software package programmed by the National Engineering Research Center for Biotechnology (Shanghai), East China University of Science and Technology (Fig. 1).

Analytical Procedures

Glucose Concentration

A glucose oxidase–peroxidase kit was applied with a spectrophotometer method.

Stability Test for Yeast Plasmid

The yeast transformant selected from the SD plate was inoculated into 2 mL of YEPD culture broth containing yeast powder, polypeptone, and glucose, and this was termed as 0th-generation cell. It was cultivated on a rotary shaker at 30°C for 24 h, and the cell was taken as 10th generation. Two microliters of the 10th-generation cell was then inoculated into 2 mL of YEPD medium, cultivated under the same conditions, and the cell obtained possessed the age of 20th generation. The cultivation was carried out continuously until the 50th generation had elapsed. From the 0th generation, a small amount of culture broth was sampled at every 10th-generation interval and spread onto the YEPD plate after appropriate dilution. Incubated at 30°C for 24 h, the colony was inoculated onto the SD

plate and incubated for 72 h. The stability of the plasmid was then calculated as follows:

Stability of plasmid at *n*th generation =
(no. of colonies grown on SD plate at *n*th generation/
no. of colonies grown on YEPD plate at *n*th generation)/
(no. of colonies grown on SD plate at 0th generation/
no. of colonies grown on YEPD plate at 0th generation)

Determination of Biologic Activity of Human IFN-α2a

Inhibition of the cell affection method *(5)* with the WISH cell/VSV system was applied, and the assay was conducted by the quality inspection laboratory of Shanghai Wanxing Bio-Pharmaceutical.

Determination of Ethanol

Ethanol was determined using gas chromatography.

Protein Concentration

Protein concentrations were determined by the Coomassie blue dye binding assay. The reagent Bio-Red was used for the color development reaction with the protein, and the OD was estimated at 595 nm.

Biomass Concentration

Being diluted the sample was determined at 600 nm. The relationship between culture turbidity and dry cell weight was established.

Dry Cell Weight

Dry cell weight was estimated by centrifuging the sample, and the cell precipitate was dried to constant weight after washing.

Results and Discussion

After construction of the recombinant strain, it is essential to explore the fermentation process systematically for enhancement of productivity and reduction of production costs. Fed-batch cultivation is an effective approach to realize the high expression of IFN-α2a.

Regular Pattern of IFN-α2a Fermentation Process

The seed cultivated in SD seed medium for 18 h was transferred into a 2.6-L jar fermentor with 5% inoculum. The fermentation was conducted at 30°C and an aeration rate 0.5 vvm. One-eighth of the whole fermentation medium was fed evenly during 10–20 h of fermentation. In the course of fermentation, the pH was adjusted around 6.30 with NH_4OH, and the broth was harvested at 36 h.

Figure 2 shows that glucose was nearly depleted around 12 h, and the OD_{600} value increased rapidly during 4–22 h of fermentation and was maintained on the whole at 14–15. DO was not restricted, because air saturation

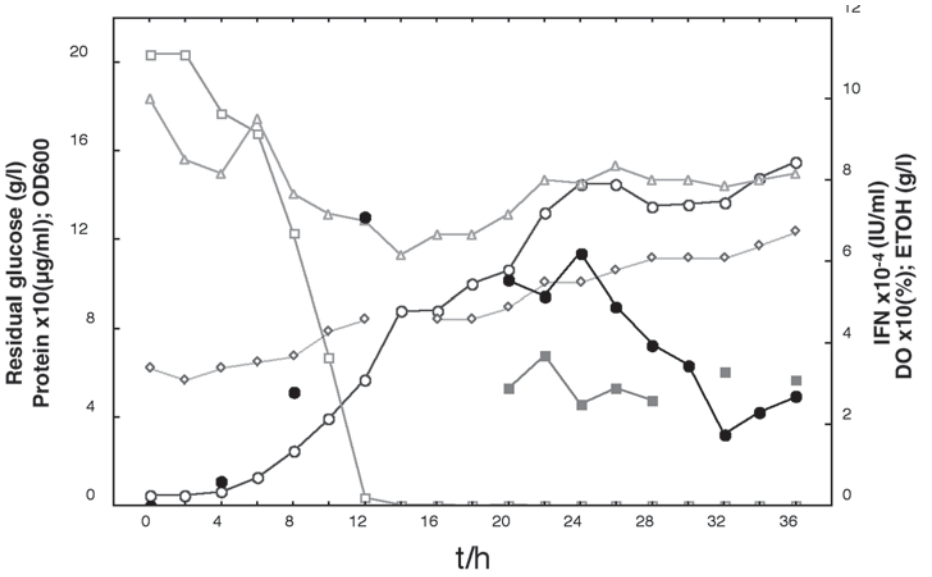

Fig. 2. Time course of conventional human IFN-α2a batch fermentation process. ○, OD_{600}; □, residual glucose; ◇, protein; △, DO; ●, ethanol; ■, IFN-α2a activity.

was >60% during the course of fermentation. Ethanol content of the fermentation broth gradually increased to 71 g/L during the growth phase (0–11 h) and was consumed gradually by the culture to about 20 g/L after entering the production phase. Overproduction of ethanol during the growth phase may be attributed to the high specific growth rate, which led to the emergence of a Crabtree effect. It is evident that a high concentration of ethanol is unfavorable to the expression of foreign protein *(7)*. Besides, no glucose was available during the production phase; instead, ethanol was used as carbon and energy source. This might also hamper the biosynthesis of interferon. Consequently, in this experiment the expression of IFN-α2a activity during 20–36 h did not increase remarkably. The activity of the broth at the end of fermentation (36 h) was 3.1×10^6 IU/mL.

Effect of Addition of Glucose on Expression of IFN-α2a

In this experiment, the culture conditions were the same as in Fig. 1, except glucose was supplemented batchwise after it was exhausted at about 14 h; that is, 0.5% glucose was added each time at about 2-h intervals when the residual glucose had nearly depleted. Figure 3 shows that appropriate supplement of glucose and the maintenance of residual glucose at a low level are favorable to the production of IFN. The reduced accumulation of ethanol resulted in channeling more carbon flux to the product synthesis. As a result, the biologic activity of IFN-α2a was enhanced to 4.9×10^6 IU/mL.

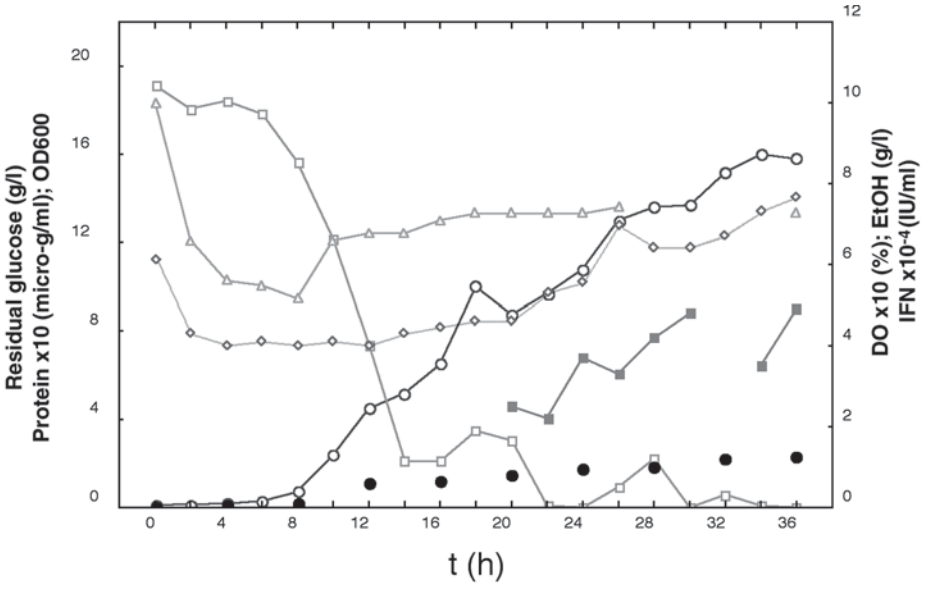

Fig. 3. Effect of addition of glucose on various state parameters during mid and late phase. ○, OD_{600}; □, residual glucose; ◇, protein; △, DO; ●, ethanol; ■, IFN-α2a activity.

Effect of Adenine Supplement on Expression of IFN-α2a

Since the genetic background of the producing strain is adenine (Ade) auxotrophic, the lack of Ade during fermentation will exert a negative impact on the growth of the recombinant yeast and the expression of foreign protein. It is suggested that a feasible Ade feeding strategy is crucial for the expression of IFN-α2a activity.

While keeping the composition of the basal medium unchanged, Ade was fed at a constant rate of 2 μg/(mL·h) during 10–20 h of fermentation, while feeding of glucose began at 13 h, when glucose was nearly exhausted, and was maintained at a low concentration until the end of fermentation. Compared with the results in Fig. 3, OD_{600} in this batch was greatly increased to 24, and the protein concentration was increased to 276 mg/L. The content of ethanol generated was also reduced tremendously during the process, and as a result, 1.3×10^7 IU/mL of biologic activity was achieved (Fig. 4).

Since Ade was favorable to the production, the effect of a higher concentration of Ade was investigated. During 10–20 h of fermentation, Ade was fed at a constant rate of 2 μg/(mL·h). The results shown in Fig. 5 indicate that in the course of fermentation, the content of ethanol was rather high, and as a result, the biologic activity harvested was 1.1×10^7 IU/mL. This indicates that the Ade feeding rate of 2 μg/(mL·h) was sufficient for the expression of IFN-α2a.

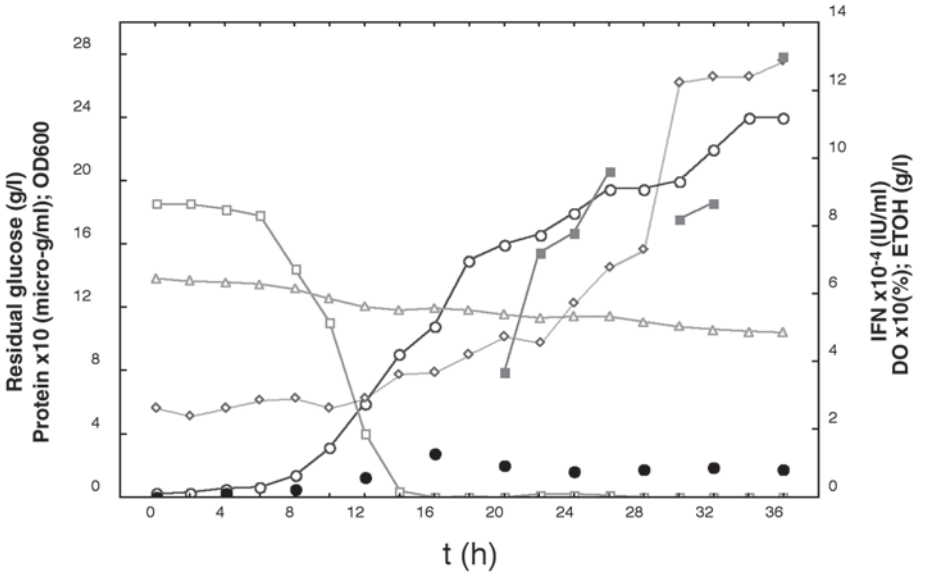

Fig. 4. Effect of feeding Ade at constant rate (2 µg/[mL·h]) on expression of IFN-α2a. ○, OD_{600}; □, residual glucose; ◇, protein; △, DO; ●, ethanol; ■, IFN-α2a activity.

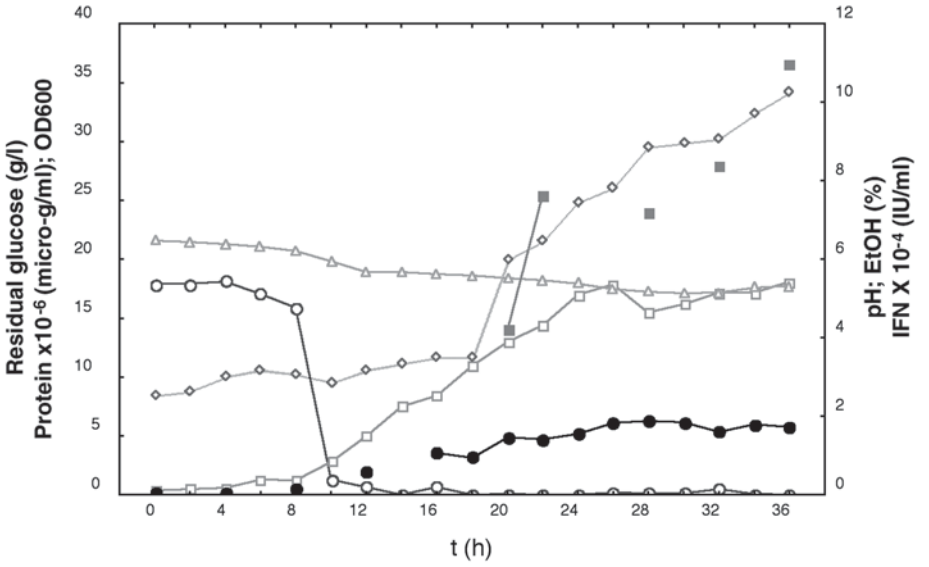

Fig. 5. Effect of feeding Ade at constant rate of 4 µg/(mL·h) on expression of IFN-α2a. ○, OD_{600}; □, residual glucose; ◇, protein; △, pH; ●, ethanol; ■, IFN-α2a activity.

When the time of the feeding of 2 µg/(mL·h) of Ade was adjusted to 10–30 h, growth was promoted, OD_{600} reached 28, but the activity was only 5.5×10^6 IU/mL. The prolongation of the feeding of Ade led to plasmid instability, and only 54% of the cells harbored the plasmid at the end of the fermentation (data not shown).

Table 1
Effect of pH on Stability and Production of IFN

PH controlled	Maximal OD_{600}	Plasmid stability (%)	IFN-α2a (IU/L) (maximal value)
5.3–5.5	20.8	80.6	13.0×10^9
5.8	20.0	61.0	9.8×10^9
6.0	17.5	58.4	6.5×10^9

Table 2
Effect of Polypeptone on Expression of IFN-α2a

Polypeptone	OD_{600}	IFN-α2a activity (IU/mL)
2.0% in basal medium; none in feed	24	1.1×10^7
2.5% in basal medium; none in feed	16.5	6.3×10^6
2.0% in basal medium; +0.5% in feed during 10–20 h	10	2.9×10^6

Effect of pH on Expression of IFN-α2a

In the course of fermentation, change in pH is dependent on the metabolic feature of the yeast, and the composition of the medium and its environmental conditions. The cell's capability to adjust the pH of its environment is limited, so its growth and the expression would be greatly affected if the environmental conditions were altered acutely.

During the expression phase (20–36 h), the pH in different batches was controlled to 5.5, 5.8, and 6.0, respectively, with ammonia, so as to determine an appropriate pH for the expression of foreign protein. The results in Table 1 show that the expression was greatly influenced by the pH in the expression phase.

Within the range of the experiments, the stability of the plasmid was reduced with the increase in pH. The plasmid stability at the end of fermentation (36 h) in batches at pH 5.5, 5.8, and 6.0 during the expression phase was 80.6, 61.0, and 58.4%, respectively, and the expression level was reduced accordingly; the bioactivity of the IFN-α2a reached 1.3×10^{10}, 9.8×10^9, and 6.5×10^9 IU/L, respectively. Consequently, the pH in the expression phase should be controlled at about 5.5.

Effect of Polypeptone on Expression of IFN-α2a

The results in Table 2 show that 2% polypeptone could meet the demand of growth and the expression of IFN-α2a, and that the increase in the supply of polypeptone in basal medium and/or feeding had an adverse effect on expression and plasmid stability.

Conclusion

To circumvent the conversion of glucose carbon to ethanol and prevent the accumulation of an inhibitory concentration of ethanol during the fermentation process of recombinant *S. cerevisiae*, glucose was fed into the medium to control the residual glucose concentration at a very low level when the glucose in the basal medium was exhausted. As a result, IFN-α2a activity was increased from 3.1×10^6 to 4.9×10^6 IU/mL. The bioactivity of IFN-α2a was further improved to 1.3×10^7 IU/mL by an Ade supplement strategy. The pH of the expression phase had an important effect on the expression level of IFN-α2a, and it should be controlled at 5.5. The addition of polypeptone had an adverse effect on the expression of IFN-α2a.

References

1. Wu, W. T., Ding, X. S., and Liu, J. J. (1996), in *Genetic Engineering for Medicine*, Wu, W. T., ed., People's Health Press, Beijing, pp. 154–161.
2. Kingsman, S. M. and Kingsman, A. J. (1983), *Symp. Soc. Gen. Microbiol.* **35,** 211–254.
3. Slocumbe, P., Easton, A., Boseley, P. , and Burke, D. C. (1982), *Proc. Natl. Acad. Sci. USA* **79,** 5455–5459.
4. Tuite, M. F., Dobson, M. J., Roberts, N. A., King, R. M., Burke, D. C., Kingsman, S. M., and Kingsman, A. J. (1982), *EMBO J.* **1,** 603–608.
5. Li, Y. (1987), *Chin. J. Biotechnol.* **3(2),** 81.
6. Huo, K.-K., Yu, L.-L., and Chen, X.-J. (1992), Sci. China, Ser. B 9:922–928.
7. Teo, W. K. and Yap, M. G. S. (1994), in *Better Living Through Innovative Biochemical Engineering*, Continental Press Pte Ltd., Singapore, pp. 357–362.